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## 9

## Certain Dyes as Pharmacologically Active Substances in Fish Farming and Other Aquaculture Products

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### 9.1 Introduction

The last 40 years have brought enormous changes to the aquaculture industry. The farming of fish and of seafood products has been continuously increasing from 3.9% by weight in 1970 to 36% in 2006 according to the World Health Organization (WHO) and Food and Agriculture Organization (FAO) of the United Nations.<sup>1</sup> The global trend of aquaculture development gaining importance in total fish supply has remained uninterrupted. Farmed food fish contributed a record 42.2% of the total 158 million tonnes of fish produced by capture fisheries and aquaculture in 2012 (Figure 9.1). This compares with just 13.4% in 1990 and 25.7% in 2000. Since 2008, Asia has been producing more farmed fish than wild catch, and its aquaculture share in total production reached 54% in 2012, when European production rose to 18% and other continents to less than 15%.<sup>1</sup> The 15 main producer countries accounted for 92.7% of all farmed food fish and seafood production in 2012. In the same period, there was a considerable intensification of seafood trading worldwide.

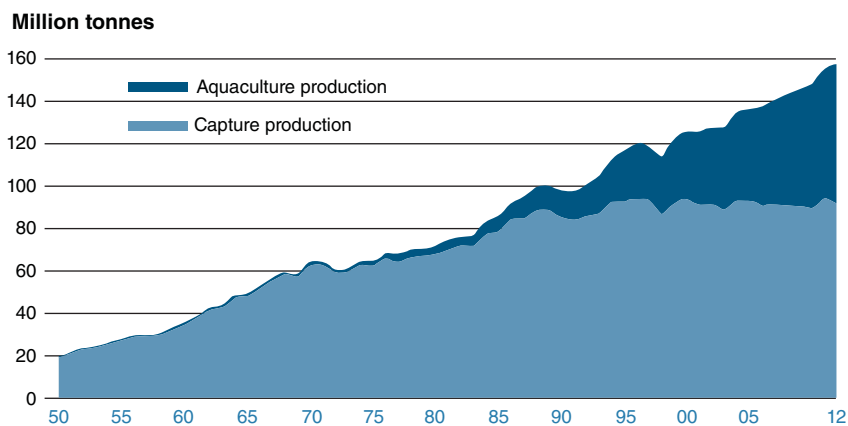
Fish is the main valued export commodity from the vast majority of the developing countries before coffee, natural rubber, cocoa, and sugar.<sup>1</sup> According to the seafood trade flows in 2010 from Natale et al.,<sup>2</sup> China appears as the major exporter to the rest of the world with also an increasing importance of Vietnam, Thailand, Chile, India, and Indonesia. China has also become the world's third largest importing country after the United States of America and Japan (Figure 9.2). The European Union (EU) is the largest market for imported fish and fishery products, and its dependence on imports is still growing. Such a food fish farming increase cannot be further intensified without controlling the zoonosanitary aspects of this agri-food industry.

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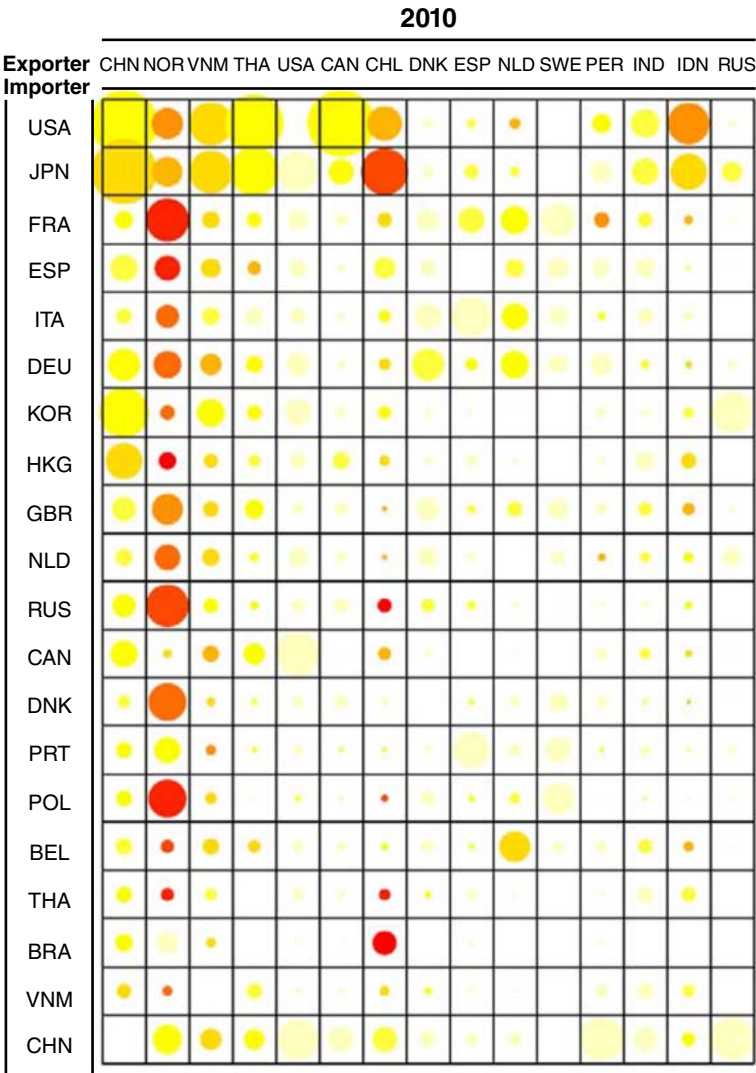
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**Figure 9.1** World capture fisheries and aquaculture production per year from FAO (2014).<sup>1</sup>

A considerable amount of food fish farming, 63% in 2012,<sup>1</sup> is now attributed to extensive and intensive freshwater inland aquaculture and also coastal brackish water ponds and shore-based mariculture. It is considered easy-to-establish aquaculture in developing countries. However, some technical barriers to trade, such as international standards and regional technical regulations in the importing countries aimed at protecting consumers from the presence of chemical residues and contaminants in traded seafood associated with intensive farming, may have significant impact on the efforts in these developing countries. For instance, disease problems have been reducing the farmed shrimp production and have forced farmers to introduce zootechnical practices and treatments to combat these diseases.

In contrast to the large therapeutic arsenal to fight against mammalian diseases, the use of pharmaceutical substances is rather limited in scope in fish and seafood farming, and it has always been basically limited to some anesthetic substances and to anti-infective and antimicrobial agents against parasitic and microbial diseases.<sup>3, 4</sup> As a consequence, the unregulated use of dye chemicals from the family of the triphenylmethane dyes, malachite green (MG), a common commercial and inexpensive fabric dye, has developed and been used as a therapeutic multi-usage drug to globally reduce parasitic, microbial, and fungal diseases found in fish and seafood farming.<sup>5</sup> MG has, for instance, been used both prophylactically and in the treatment of fungal infections for fish and eggs for more than 80 years.<sup>6</sup> In the course of the 1980s, 1990s, and 2000s, many concerns were raised in regard to the toxicity of this substance, and different toxicological studies were carried out for MG and for some other similar dyes applied or potentially applied for their therapeutic qualities in fish farming. MG has now been banned in nearly all of the regions of the world, including North America and Europe,



**Figure 9.2** International seafood trade flows from the main importers and exporters by year and origin of production (aquaculture vs fisheries). The size of the circles is proportional to the value of the exports. The shading is based on the percentage represented by aquaculture of the total fish production in the exporting country in contrast to wild catch fisheries (10 equal intervals between 0% and 100%). The lighter the grey shading, the more aquaculture; the darker the grey shading, the more product is from wild catch fisheries. Source: Natale 2015.<sup>2</sup> Reproduced with permission from Elsevier. CHN, People's Republic of China; NOR, Norway; VNM, Vietnam; THA, Thailand; USA, United States of America; CAN, Canada; CHL, Chile; DNK, Denmark; ESP, Spain; NLD, Netherlands; SWE, Sweden; PER, Peru; IND, India; IDN, Indonesia; RUS, Russia; JPN, Japan; FRA, France; ITA, Italy; DEU, Germany; KOR, Southern Korea; HKG, Hong Kong; GBR, Great Britain; PRT, Portugal; POL, Poland; BEL, Belgium; BRA, Brazil.

but can still be present in various inappropriate fish farming practices around the world.

Recently, the Joint WHO/FAO Expert Committee on Food Additives (JECFA) has evaluated the risk for public health of the use of MG<sup>7, 8</sup> and crystal (gentian) violet (CV)<sup>9, 10</sup> in fish farming. The Codex Committee on Residues of Veterinary Drugs in Foods has recommended that competent authorities should not permit their use in food-producing animals including fish/seafood farming.<sup>11, 12</sup> This should therefore lead to an absence of detectable residues in products from this industry. However, they still appear to be present, probably because they are still widely used in the textile industry and elsewhere and are commercially available as inexpensive therapeutic chemicals for ornamental fish. In addition, the dyes are persistent in the sediment of water sources for aquaculture and will be absorbed and bioaccumulated in aquaculture tissues over time.<sup>13</sup> As a result of these assessments and recommendations, several countries and the EU since 2004 have assigned a specific food safety concern to these substances and mandated that they should be actively controlled in food products and food trading derived from the fish and seafood farming industry.

There have been trade issues associated with certain dye compounds used as veterinary medicines, particularly with MG and its chemically related congeners in aquaculture. This chapter is intended to review these pharmacologically active dyes from their chemistry and toxicological concerns to their regulatory monitoring in aquaculture products due to their undesirable presence in aquaculture-sourced foods.

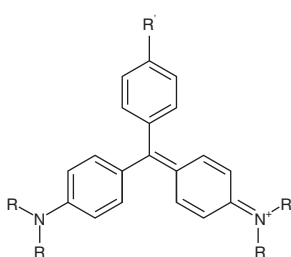
## **9.2 Therapeutic Applications and Chemistry of Certain Dyes Used in Fish Farming**

Dyes with pharmacological activity can be categorized into five chemical classes: triaryl(phenyl)methanes, phenothiazines, xanthenes, acridines, and azo compounds (Figure 9.3). In aquaculture, dyes are primarily used as a treatment for fungal and external parasite infections in fish and to protect incubating eggs from fungus. Many of the dyes described from these chemical classes have antiseptic, antimicrobial, or other medicinal properties with uses in veterinary and human medicine. Many also have unique affinities for binding to different cellular components rendering these therapeutic dyes excellent biological stains. Other dyes and pigment residues have been found in fish from environmental exposure to textile and manufacturing effluents<sup>15</sup> as well as from food additives intentionally added to color seafood products. For example, the carotenoid pigments canthaxanthin and astaxanthin are used as feed additives to redden the color of aquacultured salmon and trout flesh.<sup>16</sup> Though toxicity and safety concerns have led to restrictions and discontinuation of therapeutic dye treatments, the long history, efficacy, and ready availability of inexpensive dyes for infection control suggest that regulatory monitoring must continue.

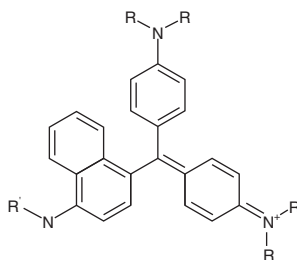
### 9.2.1 Triarylmethanes

Triarylmethane dyes are cationic and have wide application as colorants for textiles, papers, plastics, and inks and are used as biological stains. These are characterized as the structurally simple triphenylmethane dyes and the more complex triphenylnaphthylmethane structures of the Victoria blue dyes, where one phenyl ring has been substituted with a naphthyl group (Figure 9.3). The triphenylmethane dyes have a long history of therapeutic use as fungicide and ectoparasiticide agents. Gentian violet was noted to have bactericidal properties in mammalian blood in 1913,<sup>17</sup> and it is effective as a human medicine for the treatment of fungal infections of candidiasis and thrush. In 1933, Foster and Woodbury<sup>6</sup> reported MG to be unusually effective for the treatment of

#### Triarylmethanes

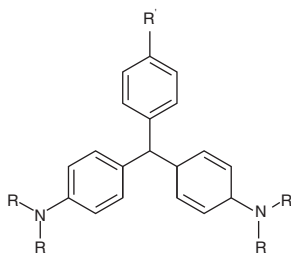


R = Me, R' = H      Malachite green  
 R = Et, R' = H      Brilliant green  
 R = Me, R' = NMe<sub>2</sub>      Crystal violet  
 R = Et, R' = NEt<sub>2</sub>      Ethyl violet  
 R = H, R' = NH<sub>2</sub>      Pararosaniline



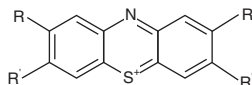
R = Me, R' = Ph      Victoria blue B  
 R = Me, R' = Et      Victoria blue R  
 R = Et, R' = Et      Victoria pure blue BO

#### Triarylmethane metabolites



R = Me, R' = H      Leucomalachite green  
 R = Me, R' = NMe<sub>2</sub>      Leucocrystal violet

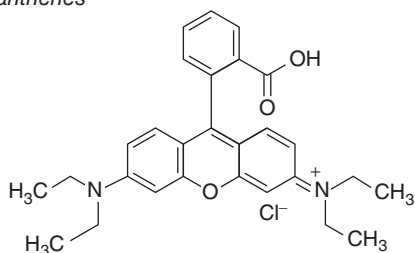
#### Phenothiazines



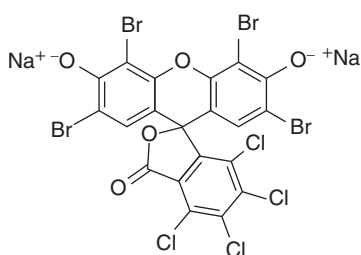
R = H, R' = NMe<sub>2</sub>      Methylene blue  
 R = H, R' = NMe<sub>2</sub>, R'' = NHMe,      Azure B

**Figure 9.3** Structures of pharmacologically active dyes. Source: Tarbin 2008.<sup>14</sup> Reproduced with permission from Elsevier.

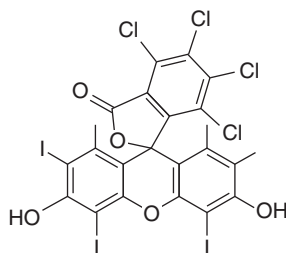
## Xanthenes



## Rhodamine B

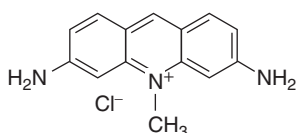


## Phloxine B

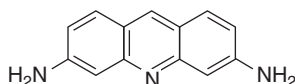


## Rose Bengal

## Acridines

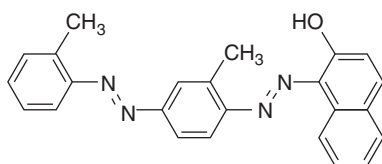


## Acriflavine

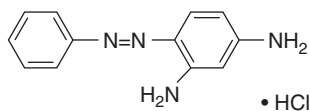


## Proflavine

## Azo Dyes



## Sudan IV



## Chrysoidine

Figure 9.3 (Continued)

fungus infections in trout, bass, and trout eggs. MG is considered to be the most effective antifungal treatment used in aquaculture.<sup>18</sup> Exposure bath treatments are effective for the control of the external protozoan *Ichthyophthirius multifiliis* in fish, and treatments of fish eggs with dilute MG effectively reduce fungal growth (e.g., *Saprolegnia*) and ensure viability of live eggs.<sup>19</sup> Other studies indicate additional members of the triphenylmethane class of compounds to

have similar antiseptic and antifungal properties. Alderman conducted *in vitro* studies of cultures of *Saprolegnia parasitica* against 11 triphenylmethane dyes and other compounds with antifungal properties.<sup>20</sup> Of the 40 compounds studied, the mercury-containing compound thimerosal and the triphenylmethane compounds MG, CV, and brilliant green (BG), monophenylrosaniline (Dahlia), and iodine green were the most effective. In a more recent study, the antifungal potency of MG, CV, BG, and methyl green was assessed against 36 different fungal strains and found to have comparable or greater activity when compared to antifungal reference standards.<sup>21</sup>

MG and metabolites are susceptible to oxidation/reduction and demethylation reactions in the presence of air and light. The MG cation has a  $pK_a$  of 6.9, and it slowly hydrolyzes to form an equilibrium mixture with the colorless carbinol base in aqueous solutions. Under acidic conditions (pH 3.5), only the cationic dye is present in solution. At pH conditions of 6.5, 7.0, and 9.0, after equilibration, the carbinol accounts for approximately 25%, 50%, and 100% of the material, respectively.<sup>22</sup> The less water-soluble carbinol has greater lipophilicity with higher potential than the cation to pass through cell walls.<sup>23</sup> After absorption, the compound is quickly metabolized to leucomalachite green (LMG). LMG is lipophilic and has a very long residence time in fatty muscle tissue. In a  $^{14}\text{C}$ -labeled study of catfish treated by a 1-hour MG exposure bath, residues bioconcentrated in the catfish at higher concentrations than the exposure bath.<sup>24</sup> Immediately after exposure, LMG residue concentration was slightly higher than MG in muscle. After 14 days, MG had decreased to the method detection limit, while concentrations of LMG in muscle were more than 40 times higher. LMG was still quantifiable in muscle 42 days later. Demethylated metabolites of LMG were also identified in catfish muscle after treatment by MG exposure bath.<sup>25</sup> Metabolized LMG in fish muscle has been observed to oxidize back to MG when fish muscle is frozen.<sup>26</sup> The complex interconversions that these compounds undergo have led to a wealth of studies in the literature to better understand the chemistry of the triphenylmethanes in aquatic species.

CV and BG are other triphenylmethane dyes with similar properties to MG. CV is hexamethyl-*p*-rosaniline (Figure 9.3), whereas the similar dye product, gentian violet, is a mixture that is primarily composed of CV and also contains methyl violet, the pentamethyl-*p*-rosaniline compound. Leucocrystal violet (LCV) is the metabolic marker residue in fish after treatment bath exposure to CV. Thompson et al.<sup>27</sup> determined the concentration of CV and LCV residues in catfish muscle following the exposure to a 1-hour treatment bath of CV (100  $\mu\text{g/l}$ ). Catfish were then returned to a pond for withdrawal studies. One hour after exposure, a CV concentration of 0.5  $\mu\text{g/kg}$  was determined, and residues of LCV in muscle were 12  $\mu\text{g/kg}$ . CV concentration quickly dropped below the detection limit, while LCV was still present at a concentration of 3  $\mu\text{g/kg}$  79 days after the treatment bath. The predominance of the leuco metabolite was also noted after low-concentration exposure bath treatment (10  $\mu\text{g/l}$ , 1 hour) of salmon and tilapia.<sup>28</sup> Chan et al. conducted depletion studies of CV and LCV in salmon.<sup>29</sup> One day after bath exposure



to CV, 98% of the residues were in the form of LCV, and this metabolite was detected in salmon as long as 91 days after exposure.

Data are more limited for the metabolism of BG, though this compound is also expected to metabolize to the leuco base in fish muscle. Leucobrilliant green (LBG) is readily oxidized to BG, limiting the stability of this compound and resulting in the lack of a commercially available standard. Andersen et al. fortified catfish muscle with LBG and found it to oxidize to BG during the extraction process.<sup>30</sup> Hurtaud-Pessel et al. identified both BG and LBG residues in samples of trout treated in a BG bath.<sup>31</sup> Immediately after bath exposure, BG and LBG residues were in equal proportion in the trout muscle. Two hours after exposure, the LBG residue concentration in muscle was two-thirds of the concentration of the BG residues. In another study from Schneider et al.,<sup>32</sup> LBG was not identified in incurred samples of salmon, catfish, and tilapia that had been exposed to a low-concentration bath (10 µg/l) of BG. These studies indicate that the parent dye is an acceptable marker residue to identify BG treatment, while regulatory testing for MG and CV must include the contribution of the leuco forms, which have greater stability and very long residence time in fish muscle.

The triarylmethane dyes Victoria blue B and Victoria pure blue BO were recently detected at low concentrations in one or two samples of wild freshwater eel, thought to be the result of dye effluents from textile plants.<sup>15</sup> Victoria pure blue BO residue was found in a sample of white fish as reported in the 2010 annual report of the European Rapid Alert System for Food and Feed (RASFF).<sup>33</sup>

### 9.2.2 Phenothiazines

Methylene blue (MB) is in the phenothiazine dye class of dyes. As the first synthetic drug, it has a long history and numerous applications for human and animal medical use. MB has been used in ruminant animals as an antidote against nitrate and cyanide poisoning.<sup>34</sup> In human medicine, it has been used to treat malaria, depression, and methemoglobinemia and is under current investigation to slow neurodegenerative disease.<sup>35</sup> In aquaculture, MB is effective as an anti-septic and disinfectant, with similar indications for use as MG against *I. multifiliis* and to protect fish eggs from fungal infestation, though with lower efficacy than MG.

Several studies noted that the uptake of MB residues into fish muscle was much lower than residues of triphenylmethane dyes under similar exposure conditions.<sup>36, 37</sup> In studies of catfish subjected to MB treatment baths, fish were exposed to 1 or 5 mg/l of MB for 1 hour. The average concentration of MB found in the muscle of these catfish was 10 µg/kg or less for the lower exposure group and 16 µg/kg for the higher exposure.<sup>37</sup> Like the triphenylmethane dyes, MB is expected to quickly metabolize to a colorless leuco form, though it may not be possible to stabilize and isolate the leuco form from the muscle.<sup>38</sup> Turnipseed et al. documented the instability of this compound in studies of incurred catfish

muscle, noting that leucomethylene blue (LMB) readily oxidizes back to MB.<sup>37</sup> In the metabolic process, MB may also lose one, two, or three methyl groups to form the demethylated azure dye metabolites or fully demethylate to thionine. Thionine was reported to be a protein-bound conjugate with a long residence time in milk from treated dairy cows.<sup>34</sup>

### 9.2.3 Xanthenes

Xanthene dyes consist of compounds such as fluorescein, rhodamine, and eosin. Compounds from this class are commonly used as fluorescent biological stains and as laser dyes. Rhodamine compounds and fluorescein have been used in tracer studies to monitor the flow of water in rivers and aquatic systems.<sup>39</sup> For example, these dyes were added to pesticide formulations used in sea lice treatment to follow the dispersion of pesticides to surrounding environmental waters.<sup>40, 41</sup> Some dyes from this class have bactericidal, insecticidal, or fungicidal properties.<sup>42</sup> Rhodamine B and the halogenated derivatives Rose Bengal and phloxine B showed antifungal action against *S. parasitica* in culture studies by Alderman.<sup>20</sup> Some dyes from this class act as photosensitizing insecticides. Xanthenes have been formulated for uptake by insects, where they are photoactivated by sunlight to form cytotoxic singlet oxygen and other reactive species.<sup>43</sup> The halogenated eosins (e.g., Rose Bengal, erythrosine, etc.) are effective in this regard. Phloxine B has been commercially developed as a photosensitizing insecticide used to control fruit flies in animal feed. Blair proposed the use of phloxine B to treat the protozoan infection *I. multifiliis* in fish.<sup>44</sup> In this application, phloxine B would be added to an aquaculture pond at night, absorbed by protozoa, and then activated by sunlight to generate free radical species to kill the protozoans. In another study, singlet oxygen produced from the irradiation of Rose Bengal was found to be effective against the virus responsible for white spot syndrome in *kuruma* shrimp populations.<sup>45</sup> Though there may be potential for xanthene residues to be present in seafood either by aquaculture or pesticide use or by the use of these compounds as color additives, reports of their identification in regulated products were not found.

### 9.2.4 Acridines

Acridine dyes were originally isolated from coal tars and were introduced as an antiseptic in 1912. Acridine dyes such as acriflavine, proflavine, and quinacrine have antiseptic properties with medicinal uses to treat malaria, sleeping sickness, and giardiasis.<sup>46</sup> Reported uses in veterinary medicine are the treatment of mastitis, urinary or enterobacterial infections, and parasite infections.<sup>34</sup> Though not as effective as MG, acriflavine is prescribed for use as a mixture with proflavine to treat external fungal infection in aquarium fish and to disinfect fish eggs.<sup>47</sup> Plakas et al.<sup>47, 48</sup> found acriflavine and proflavine to be poorly absorbed into the muscle

of catfish after bath treatment; Yu et al.<sup>49</sup> found similar results for trout. Glucuronosyl and acetyl conjugates were identified as the metabolites of proflavine in trout and catfish, yet the parent compounds were the primary residues present in muscle. The elimination half-life for catfish muscle was 1.5 days for proflavine and 5.3 days for acriflavine.<sup>47</sup> Residue concentrations in the skin remained largely unchanged 14 days after exposure bath treatment.

### 9.2.5 Azo Dyes

While many azo dyes are regulated in foods as illegal color additives (e.g., Sudan dyes), azo dyes such as Sudan IV (scarlet red) and Congo red are active against Gram-negative bacteria.<sup>34</sup> The azo dye chrysoidine was isolated in 1914 and found to have high bactericidal activity.<sup>50</sup> Chrysoidine was reportedly used to color lower-quality fish to look like more expensive yellowfin tuna.<sup>51</sup> Reyns et al. reported that chrysoidine has also been used illegally to disinfect fish skin and residues of this compound should be monitored to detect abuse.<sup>52</sup>

## 9.3 Toxicological Issues

The pharmacologically active dyes considered in this chapter are prohibited from use in food-producing animals due to their toxicity and potential to cause changes in genetic material. A number of studies have been conducted over several decades to classify the effects of these compounds on aquatic and mammalian species. Not every compound has been studied in depth, but similarities within the structures may be used as the basis to predict similar toxicological effects. In some cases, individual study results have been summarized in larger risk assessment (RA) evaluations. Comprehensive toxicological studies and summaries by the International Agency for Research on Cancer (IARC), the US National Toxicology Program (NTP), the JECFA, the European Food Safety Authority (EFSA), and the European Medicines Agency (EMA) are briefly summarized in the following text for specific classes of dyes.

### 9.3.1 Triarylmethanes

The health effect of MG has been studied extensively, with comprehensive toxicology review articles published,<sup>53, 54</sup> several major animal studies, and recent evaluations by international consortia. The toxicology and carcinogenicity of MG and LMG were investigated by the NTP and summarized in two technical reports.<sup>55, 56</sup> *In vitro* studies did not show either compound to be mutagenic.<sup>55</sup> However, 2-year feeding studies with rats and mice showed that MG caused an increase in tumor formation in female rats and that LMG was more potent, causing an increase in cancer in all rats and female mice<sup>56, 57</sup>. These results were consistent with other studies, where tumors were observed in *in vivo*

studies,<sup>58</sup> but *in vitro* assays with bacterial and human cell lines showed MG to be cytotoxic, whereas LMG did not cause mutations.<sup>59,60</sup> *In vitro* studies indicated that mammalian and human intestinal microflora efficiently convert MG to LMG.<sup>61</sup> In the livers of treated rats, additional demethylated and N-oxide metabolic products were observed, indicating that *in vivo* enzyme activation may be necessary for more severe genotoxic or mutagenic effects.<sup>62</sup>

CV toxicology has also been reviewed.<sup>63</sup> Littlefield studied mice exposed to CV and determined a no-observed-effect exposure level that would prevent formation of liver tumors.<sup>64</sup> Safe doses were indicated to be 1–2 µg/kg. Like MG, human and mammalian intestinal microflora reduced CV to LCV in *in vitro* studies.<sup>65</sup> Genotoxic and mutagenic effects have been observed for other triarylmethane dyes as well. *In vitro* assays of BG, methyl violet, and Victoria blue indicated mutagenicity with fungal yeast cells.<sup>66</sup> Pararosaniline and other triphenylmethane compounds comprising magenta dye have been designated class 1 carcinogens by the IARC.<sup>67</sup>

Trout eggs and pregnant rabbits exposed to MG yielded significant abnormalities to the developing offspring.<sup>68</sup> Teratogenicity studies have been conducted for CV as well.<sup>69</sup> In fish, lethal concentration (LC<sub>50</sub>) values have been determined for MG in different fish and range from 0.5 to 5.6 mg/l.<sup>70,71</sup> For CV, LC<sub>50</sub> was 0.2 mg/l.<sup>71</sup>

More recently, the JECFA evaluated the risk of using MG and CV in fish farming on public health.<sup>8,10</sup> After reviewing studies on the genotoxic effects of these dyes and metabolites, the committee did not support permitting MG or CV use in food-producing animals and decided it inappropriate to establish acceptable daily intake (ADI) values for these compounds. Full toxicological evaluations on these compounds were published recently by the WHO.<sup>7,9</sup>

### 9.3.2 Phenothiazines

In NTP studies,<sup>72</sup> MB trihydrate was found to be genotoxic in bacterial assays and to produce some evidence of carcinogenesis in male rats and mice. Anemia and a decreased ability of blood to bind oxygen (methemoglobinemia) were also observed in high-dose groups of rats and mice during the 2-year study. Reproductive toxicological effects have been noted as well.<sup>73</sup> The IARC provided a thorough summary of MB information and toxicological studies in the 2015 *Monograph*.<sup>74</sup> DNA damage from singlet oxygen or free radicals was observed when MB use was combined with white light photoactivation, but genotoxic effects have not been described for *in vivo* studies without photoactivation.<sup>74</sup> MB was designated as class 3, or not classifiable for carcinogenicity in humans<sup>74</sup>. The azure dye metabolites of MB were found to be mutagenic in bacterial assays.<sup>72</sup>

In a study of direct toxicity to fish, the 24-hour LC<sub>50</sub> for MB fish exposure was 25 times higher than the more toxic MG (18 vs 0.6 mg/l).<sup>71</sup> MB has been studied extensively for use in human medicine. With human oral and intravenous

dosing at much higher than residue concentrations, some toxicity has been noted, particularly with respect to adverse effects in the blood.<sup>35, 74</sup> The EMA published a report on the safety of MB for use as a human drug to reverse methemoglobinemia from drug and chemical poisonings.<sup>73</sup>

### 9.3.3 Xanthenes

The toxicity of rhodamine dyes has been studied by the IARC and the NTP. The IARC<sup>75, 76</sup> reported that rhodamine B and 6G were carcinogenic to rats in subcutaneous exposure studies. The NTP<sup>77</sup> prepared a technical report based on rhodamine 6G feeding studies, where equivocal evidence of carcinogenicity was found in rats, but no evidence was found for mice. EFSA<sup>78</sup> concluded that rhodamine B is potentially genotoxic and carcinogenic. Rowiński and Chrzanowski<sup>79</sup> summarized differences in toxicity between two xanthene dyes used as aquatic tracers – rhodamine B and rhodamine WT – where the latter was designed to have lower biological adsorption and lower toxicity. In fairy shrimp, the 24-hour lethal concentration ( $LC_{50}$ ) of rhodamine WT was approximately 200 times higher than for rhodamine B.

Phloxine B (D&C Red No. 28) has been approved in the USA as safe to use as a color additive for some cosmetic products and drugs.<sup>80</sup> Due to the potential of this and other halogenated fluorescein dyes (e.g., Rose Bengal) to form reactive oxygen species after the dyes are activated with light, additional toxicology evaluations have been performed to investigate genotoxicity after light exposure.<sup>81</sup> DNA damage has been reported for bacteria and human skin cell exposure to phloxine B and light from a fluorescent bulb.<sup>81</sup> Redness and swelling were observed after Rose Bengal application to damaged skin with exposure to visible light and sunlight.<sup>81</sup>

Toxicological effects in fish by xanthene dyes were described by Tonogai et al.<sup>71</sup> The  $LC_{50}$  for rhodamine B was 25 times higher than the more toxic MG (17 vs 0.6 mg/l), but rhodamine B had a much higher octanol–water partition coefficient suggesting better efficiency for permeating cell membranes ( $K_{ow} = 74$  vs 5.6). Halogenated xanthene dyes were also evaluated in this study of Himedaka fish.  $LC_{50}$  values (24 hour) were 130, 280, 710, and 1000 mg/l for Rose Bengal, phloxine B, erythrosine, and eosin, respectively.<sup>71</sup>

### 9.3.4 Acridines

Available information on the acriflavine–proflavine mixture acriflavinium chloride was reviewed by the IARC<sup>82</sup> in 1977, though at the time there was not enough toxicological data available to draw conclusions about carcinogenicity. Proflavine salts were evaluated in 1980 and were observed to be genotoxic in viral and bacterial assay.<sup>83</sup> These planar compounds can intercalate between DNA base pairs and cause frame shift and other types of mutations.<sup>84</sup>

### 9.3.5 Azo Dyes

The IARC has reported on the carcinogenicity of several Sudan and azo dyes.<sup>85</sup> Sudan I was determined to be carcinogenic based on oral dosing studies in rats and genotoxic in *in vitro* studies.<sup>86</sup> By their structural similarity to Sudan I, other Sudan dyes are considered to be potentially genotoxic and carcinogenic.<sup>78</sup> Potentially carcinogenic aromatic amine metabolites are formed from the Sudan dyes when the azo bond is reduced by human intestinal microflora and liver enzymes.<sup>87</sup>

Chrysoidine was found to have high acute toxicity to fish with a 24-hour  $LC_{50}$  of 0.5 mg/l and was predicted to easily permeate gills based on a high octanol–water partition coefficient.<sup>71</sup> Bladder cancer in humans has been reported after long-term exposure to chrysoidine, though insufficient data are available to classify chrysoidine as a carcinogen (IARC class 3).<sup>88</sup> This dye was reported to be mutagenic to bacteria and to produce tumors and leukemia in mice.<sup>88</sup>

## 9.4 Regulatory Issues

To prevent the risk for human consumers from unexpected amounts of toxic chemicals possibly found in traded aquaculture products, a significant number of countries across the world have introduced regulations into their “food safety” laws. Toxicologically based limits called maximum residue limits (MRLs) have been set for approved drugs in seafood as well as in other food products from animal origin.<sup>89–91</sup> These MRLs are based on ADIs established after human food safety RAs.<sup>92, 93</sup>

Internationally, the WHO and the FAO have also derived such risk management (RM) recommendations (MRLs) acknowledged through the Codex Alimentarius General Standard for Food Additives (GSFA)<sup>94</sup> and posted in the GSFA database: <http://www.fao.org/fao-who-codexalimentarius/standards/gsfa/en/>. Over a period of more than 50 years, these internationally recognized MRLs have been derived for a certain number of food additives. This includes veterinary drug chemicals as a follow-up of the human food safety RAs and operated under the auspices of the WHO and FAO by means of the Codex Committee on Residues of Veterinary Drugs in Foods, acting as the risk manager, based on RAs prepared by an independent scientific committee, the JECFA.<sup>95</sup>

Regionally, many countries have aligned their food safety laws with the RA and RM recommendations of the internationally recognized WHO/FAO. This is the case for a majority of Asian, African, and Latin American countries. Moreover, a few countries, in cooperation with the WHO/FAO, have also implemented their own process of RA and RM by means of funding their own national Food Safety Agencies and collaborating with their government departments responsible for public health, agriculture, and fisheries.

For instance, in the USA, the Food and Drug Administration (FDA) is the regulatory body having the mandate for both RA and RM issues for veterinary drug use in seafood.<sup>92, 96</sup> For Canada, according to the *Food and Drug Act*, Health Canada through its Health Products and Food Branch (HPFB) is the administration concerned with both RA and RM for all food safety issues.<sup>97, 98</sup> In the EU, according to the General Food Law Regulation (EC) No. 178/2002, it is the Directorate-General of the European Commission for Health and Food Safety (DG-SANTE) that is in charge of the RM issues in coordination with the 28 EU Member States' regulatory competent authorities.<sup>99, 100</sup> In addition, the EMA<sup>101, 102</sup> and the EFSA<sup>103, 104</sup> are the two EU regulatory bodies in charge of the RA issues for residues of human and veterinary medicinal products and for all the other chemical residues and contaminants, respectively.

For Japan, the Pharmaceutical and Food Safety Bureau of the Ministry of Health, Labour and Welfare (MHLW) is the regulatory body in charge of both RM and RA issues.<sup>105, 106</sup> Since 1991, in Australia and New Zealand, there has been a bi-national food safety agency called the Food Standards Australia New Zealand (FSANZ) administration in charge of the joint Food Standards Code,<sup>107, 108</sup> which lists requirements for foods such as additives, food safety, labeling, and genetically modified foods. They share with the Australian Pesticides and Veterinary Medicines Authority (APVMA) the responsibilities for setting MRLs. All the RM issues in terms of enforcement and interpretation of the Code are the responsibility of the state and territory departments and food agencies within Australia and New Zealand.

For the Russian Federation, to enforce the federal laws on the quality and safety of food products and the sanitary and epidemiologic rules and regulations (San-PiN), the Federal Service for Surveillance on Consumer Rights Protection and Human Well-Being (Rospotrebnadzor)<sup>109, 110</sup> is the federal executive authority in charge of the RAs and other activities linked to the implementation of control and supervision in the sphere of sanitary and epidemiological well-being of the population. The Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor)<sup>111, 112</sup> under the Ministry of Agriculture (MoA) is the federal organization of executive power, carrying out RM functions on control and supervision in the field of veterinary science including aquatic biological resources.

For China, the MoA<sup>113, 114</sup>, the National Health and Family Planning Commission (NHFPC),<sup>115</sup> the General Administration of Quality Supervision, Inspection and Quarantine (AQSIQ),<sup>116, 117</sup> the State Food and Drug Administration (SFDA),<sup>118</sup> and the Commerce Department share the responsibilities for the food safety RM. However, the RA issues have been covered by the National Center for Food Safety Risk Assessment (CFSA)<sup>119</sup> since 2011.

When specifically looking at seafood safety and considering the veterinary drugs approved in aquaculture in the various regions of the world, it is obvious there are very few of these veterinary chemicals that have been effectively addressed with an RA to finally receive an official authorization with an MRL and consequently a registered use as a veterinary medicine treatment in aquaculture.

When the drug has not been approved after its RA or if a drug has not been assigned an MRL or ADI, then the substance is considered not safe at any concentration for humans and is prohibited from use in animal production. There is a “zero tolerance” concern for prohibited veterinary drugs in seafood, where “zero” is at or near the limit of detection of the analytical equipment in place for the official control. When referring to the specific internationally recognized RAs addressing the two triphenylmethane chemical products, MG<sup>8</sup> and CV,<sup>10</sup> these two substances have entered the group of non-authorized compounds to be avoided in food-producing aquaculture. The national/international regulations in place for these two pharmacologically active but undesirable substances in seafood are described in Table 9.1. Currently, the analytical - “zero tolerance” concentration in national seafood inspection programs for these two substances and for their respective leucobase metabolites ranges from 1 to 2 µg/kg, depending on the food safety RM enforced in the country of interest. Apart from these two substances, there is no other dye of concern in most of the official monitoring programs even though all are also considered undesirable. Most of the regulations across the world state that non-fully authorized drugs are thus prohibited for use in food-producing animals. However, recently the interest in other potential pharmacologically active dyes is starting to be addressed by several reference laboratories worldwide with the development of analytical methods for controlling other dye residues in seafood.<sup>14</sup> In the early 2010s, for instance, the competent authorities of a few Member States of the EU and the USFDA have started introducing analytical procedures capable of monitoring BG, Victoria blue, or MB in combination with MG and CV monitoring programs. In the EU, a new RA from EFSA is pending<sup>120</sup> for a set of aquaculture dyes with the objective of reconsidering the need to enforce new toxicologically based regulatory limits of action called Reference Point for Action (RPA). Also under consideration is an RM issue to generalize expanding the official monitoring for the presence of other dyes such as CV and BG at least.

## 9.5 Analytical Methods for Residue Control

Analytical methods to determine the presence of illegal pharmacological dyes in edible seafood products must meet a number of requirements for regulatory food control. Methods must be sensitive enough to permit residue detection at regulatory performance limits. Methods must be selective enough to provide adequate isolation of the dye residues from the complex and fatty fish matrix. Finally, methods must permit analysis of the correct metabolic marker for these dyes. Quantitative determination of residue concentration and the ability to confirm the identity of detected residues are important features of successful regulatory analysis, though these features are typically defined within the intended scope of the method, be it designed for rapid screening of many samples, accurate concentration determination, or identification with mass spectrometry.



**Table 9.1** Overview of specific regulations for dyes in aquaculture products by countries.

Specific regulations for dyes in food products worldwide								
Country/institution		Malachite green	Leucomalachite green	Crystal (gentian) violet	Leucocrystal (gentian) violet	Brilliant green	Leucobrilliant green	Other dyes potentially regulated: methylene blue, Victoria blue, etc.
Codex Alimentarius (WHO/FAO)	RM <sup>a)</sup>	CAC-MRL-RMR 2-2015		CAC-RMR 3-2016 pending		Currently none		None
	RA <sup>b)</sup>	JECFA evaluation: 70th meeting (2008) JECFA		JECFA evaluation: 79th meeting (2015) JECFA				
	RVC <sup>c)</sup>	Malachite green not to be used in food-producing animals		Gentian violet not to be used in food-producing animals				
European Union (DG-SANTE; EMA; EFSA)	RM	Prohibited substance under Decision 2004/25/EC		Non-authorized substance in line with Decision 2004/25/EC <sup>c)</sup>		Non-authorized substance in line with Decision 2004/25/EC <sup>d)</sup>		None
	RA	New EFSA RA pending for RPA <sup>d)</sup>		New EFSA RA pending for RPA <sup>d)</sup>		New EFSA RA pending for RPA <sup>e)</sup>		
	RVC	Policy of “zero tolerance” with MRPL: 2 µg/kg		Policy of “zero tolerance”; detection capability recommended by EU CRLs: 2 µg/kg <sup>f)</sup>		Policy of “zero tolerance”; detection capability recommended by EU CRLs: 2 µg/kg <sup>f)</sup>		
United Kingdom (DEFRA; FSA)	RM	Prohibited substance under Decision 2004/25/EC		Non-authorized substance in line with Decision 2004/25/EC <sup>d)</sup>		Non-authorized substance in line with Decision 2004/25/EC <sup>d)</sup>		None
	RA	New EFSA RA pending for RPA <sup>e)</sup>		New EFSA RA pending for RPA <sup>e)</sup>		New EFSA RA pending for RPA <sup>e)</sup>		

	RVC	Policy of “zero tolerance” with MRL: 2 µg/kg	Policy of “zero tolerance” with action limit: 0.5 µg/kg	Policy of “zero tolerance” with action limit: 0.5 µg/kg	
USA (USFDA)	RM	USFDA FFDCa Section 512 (21 U.S.C. 360b)	USFDA FFDCa Section 512 (21 U.S.C. 360b)	USFDA FFDCa Section 512 (21 U.S.C. 360b)	USFDA FFDCa Section 512 (21 U.S.C. 360b)
	RA	USFDA	USFDA	USFDA	USFDA
	RVC	Policy of “zero tolerance”: detection capability required: 1 µg/kg	Policy of “zero tolerance”: detection capability required: 1 µg/kg	Policy of “zero tolerance”: detection capability required: 1 µg/kg	Policy of “zero tolerance” for any dye
Canada (Health Canada, CFIA, Env Can, DFO)	RM	Canadian Shellfish Sanitation Program (CSSP)	CSSP		None
	RA	Health Canada	Health Canada		
	RVC	Policy of “zero tolerance”: 1 µg/kg	Policy of “zero tolerance”: 1 µg/kg		
Japan (MHLW)	RM	Food Sanitation Act – Article 11 MHLW Notification No. 645, 2006	Food Sanitation Act – Article 11 MHLW Notification No. 645, 2006		None
	RA	MLHW	MLHW		
	RVC	Policy of “zero tolerance”: 2 µg/kg	Policy of “zero tolerance”: 2 µg/kg		
Australia – New Zealand (FSANZ)	RM	FSANZ Standards Code	FSANZ Standards Code – Update July 2014		None
	RA	FSANZ 2005	FSANZ 1994		

(continued)

Table 9.1 (Continued)

Specific regulations for dyes in food products worldwide							
Country/institution	Malachite green	Leucomalachite green	Crystal (gentian) violet	Leucocrystal (gentian) violet	Brilliant green	Leucobright green	Other dyes potentially regulated: methylene blue, Victoria blue, etc.
Russian Federation (Rospotrebnadzor; Rosselkhoznadzor)	RVC	Policy of “zero tolerance”: 0 µg/kg	Policy of “zero tolerance”: 0 µg/kg				
	RM	SanPiN 2.3.2.1078-01	SanPiN 2.3.2.1078-0				None
	RA	Rospotrebnadzor	Rospotrebnadzor				
	RVC	No value announced	No value announced				
China Mainland (MoA/AQSIQ)	RM	China Food Additive Regulation – Banned since 2002 under Export Oriented Scheme according to exported countries’ legislations					
	RA	Refer to JECFA					
Hong Kong (CFS)	RVC	Policy of “zero tolerance”: 0 µg/kg					
	RM	Part V of the Public Health and Municipal Services Ordinance (Cap. 132) – Harmful Substances in Food Regulations – Regulation 3A					None

India (MPEDA/EIC)	RA	Refer to JECFA	None
	RVC	Policy of “zero tolerance”; 0 µg/kg	
	RM	According to exported countries’ legislations such as EU Directive 96/23/EC and Decision 2004/25/EC	
Bangladesh (MOFL/DOF)	RA	Refer to JECFA	None
	RVC	Policy of “zero tolerance”; 2 µg/kg	
	RM	According to EU Directive 96/23/EC	
	RA	Refer to JECFA	
Brazil	RVC		None
	RM	Normative Instruction SDA No. 13, (July, 15) 2015	
	RA	Refer to JECFA	
	RVC	Policy of “zero tolerance”; 2 µg/kg	
	RM	Normative Instruction SDA No. 13, (July, 15) 2015	
Brazil	RA	Refer to JECFA	None
	RVC	Policy of “zero tolerance”; 2 µg/kg	
	RA	Refer to JECFA	
	RVC	Policy of “zero tolerance”; 2 µg/kg	

(continued)

**Table 9.1** (Continued)

Specific regulations for dyes in food products worldwide								
Country/institution	Malachite green	Leucomalachite green	Crystal (gentian) violet	Leucocrystal (gentian) violet	Brilliant green	Leucobright green	Other dyes potentially regulated: methylene blue, Victoria blue, etc.	
Chile (Sernapesca)	RM	SENASA instructions	SENASA instructions					
	RA	Refer to JECFA	Refer to JECFA					
	RVC	Policy of “zero tolerance” since 1997	Policy of “zero tolerance”					
Argentina (SENASA)	RM	SENASA instructions					None	
	RA	Refer to JECFA						
	RVC							
Costa Rica (SENASA)	RM	La Gaceta n°160 - 2008					None	
	RA	Refer to JECFA						
	RVC	Policy of “zero tolerance”: 2 µg/kg						

- a) RM: risk management.
- b) RA: risk assessment.
- c) RVC: recommended value for control purposes.
- d) RM and value recommended in line with Decision 2004/25/EC.
- e) RPA: reference point for action.
- f) A risk assessment (RA) pending by EFSA.

### 9.5.1 Procedures to Extract and Analyze Triphenylmethane Dye Residues in Fish and Shellfish Muscle

In 1983, Poe and Wilson<sup>26</sup> reported that frozen muscle from fish previously treated in an MG bath would develop a green surface color on the muscle tissue. Prior to this, it was believed that MG was not absorbed by fish muscle. These authors performed the first muscle extraction using methanol and chloroform with separation of the green color from lipids on a silica column. The green extract was analyzed by infrared and absorbance spectroscopy and matched the spectra of MG standards.<sup>26</sup> This was the beginning of many studies to understand tissue uptake, metabolism, and elimination of dye residues from fish muscle. Many analysis methods for fish were developed in the late 1980s and 1990s for separation of residues by HPLC and visible absorbance detection of the intensely colored dyes. The green-blue MG and BG absorb strongly at 618 and 627 nm, respectively, while purple CV absorbs at 588 nm; all wavelengths are far from many interfering compounds. Early extraction methods were based on solvent extraction under acidic conditions to ensure that the dye–carbinol equilibrium would be shifted to the dye form<sup>121–124</sup>. Later methods incorporated procedures to detect the residue contribution of the primary leuco metabolites.

Bauer et al.<sup>125</sup> introduced a procedure in 1988 to oxidize half of a trout extract with lead oxide, sequentially analyze both portions by HPLC-VIS, and then determine the contribution of LMG by difference. Addition of lead oxide to acetonitrile–perchloric acid extracts was also used by Dafflon et al.<sup>126</sup> Roybal and Munns<sup>127</sup> developed a chromatographic analysis for simultaneous determination of CV, LCV, demethylated metabolites, and MB with electrochemical detection rather than by absorbance measurement. This technique was applied to analyze chicken muscle with acetate buffer (pH 4.5) and acetonitrile extraction, liquid partitioning into dichloromethane, and subsequent solid-phase clean-up using alumina and carboxylic acid (CBA) weak cation exchange extraction cartridges.<sup>128</sup> Allen and Meinertz<sup>129</sup> demonstrated the feasibility of introducing a post-separation reaction column based on lead oxide oxidation to permit simultaneous HPLC-VIS analysis of MG, LMG, CV, and LCV. The PbO<sub>2</sub> post-column oxidation column formed the basis of dye and leuco analysis by HPLC-VIS for the next 15 years, with a variety of procedures for dye and leucobase extraction with acid or acidic buffer and organic solvent. Fink and Auch<sup>130</sup> demonstrated the success of the PbO<sub>2</sub> column to analyze MG, CV, BG, and leuco compounds in trout extracts. Allen et al.<sup>131</sup> mixed ground trout muscle, fry, and eggs with anhydrous sodium sulfate, prepared a matrix solid-phase desorption column, and extracted MG and LMG from the column with 1% acetic acid and methanol. The extract was cleaned up by partitioning into chloroform. Hajee and Haagsma<sup>132</sup> extracted LMG and MG from eel plasma with methanol, citrate buffer (pH 3), and ascorbic acid followed by SPE with sulfonic acid cartridges.

In 1995, Roybal et al.<sup>133</sup> developed a method for LMG and MG in catfish similar to the researcher's earlier electrochemical method for CV residues with a few

additions. Hydroxylamine hydrochloride (HAH) was introduced to the acetate buffer–acetonitrile extraction solution to prevent conversion of MG to LMG in the presence of fish enzymes. *para*-Toluenesulfonic acid (*p*-TSA) was included to serve as a counterion for the cationic MG, and alumina was dispersed into the extraction mixture to adsorb fat from the extract. Residue isolation was achieved with liquid-phase partitioning into dichloromethane and SPE with alumina and propylsulfonic acid cartridges. This procedure was used for pharmacokinetic and metabolism studies of LMG and MG in catfish,<sup>24, 134</sup> for CV and LCV residue determination in catfish<sup>135</sup>, and also for a combined determination of MG, LMG, CV, and LCV in catfish and trout,<sup>136</sup> forming the basis of many later methods. For example, confirmatory analyses of dye and leuco compounds in fish were developed using particle beam LC-MS,<sup>137</sup> GC-MS,<sup>138</sup> and isotope dilution LC-MS<sup>25, 139</sup> to permit selected ion monitoring of molecular and fragment ions.

In another analytical approach to distinguish dye and leuco contributions, extracts were separated by HPLC with column effluent flowing through an electrochemical cell, diode array detection cell, and fluorescence cell.<sup>140</sup> In this procedure, MG and CV were detected by visible absorbance at 588 nm ( $\lambda_{\text{max}} = 618$  and 588 nm, respectively), while LMG and LCV were detected by fluorescence emission at 360 nm with excitation at 265 nm. To confirm the identity of the residues, two injections of each extract were made – one with the electrochemical cell off to yield the expected absorbance and fluorescence signals and the subsequent injection with the electrochemical cell on to oxidize the leuco compounds to dyes. In the latter case, the fluorescence signal at the leuco retention time would drop to baseline, and the absorbance signal at the leuco retention time would increase. Similar analysis procedures were used by Mitrowska et al.<sup>141</sup> for simultaneous determination of MG and LMG by HPLC-VIS/FL without lead oxide oxidation and by Halme et al.<sup>142, 143</sup> for LC-MS/MS analysis with and without post-column oxidation.

In 2005, the Roybal extraction was simplified, and an *in situ* oxidation procedure was incorporated into the extraction procedure to convert leucobase to dye with the addition of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).<sup>144</sup> This permitted sensitive analysis of the sum of MG and LMG in a variety of seafood products with HPLC-VIS and quantification and confirmation of residue identity by LC-MS<sup>n</sup> with no-discharge atmospheric pressure ionization at and below concentrations of 1 µg/kg for complete regulatory monitoring.<sup>145, 146</sup> The method was later extended to include CV, LCV, and BG residues<sup>30</sup> and adapted for other analytical procedures including LC-MS/MS analysis.<sup>147</sup>

Though extract clean-up procedures for triphenylmethane compounds often include similar procedures based on acid/organic solvent extraction with partitioning into dichloromethane and cation exchange SPE cartridge clean-up, many variations exist. Tarbin et al.<sup>148</sup> developed procedures to extract trout with citrate buffer (pH 4), sodium chloride, and acetonitrile. Analysis was by HPLC-VIS and electrospray ionization LC-MS, both following post-column oxidation with lead oxide. Bergwerff et al.<sup>149</sup> extracted trout with

McIlvaine buffer (pH 3, citric acid/disodium hydrogen phosphate buffer), *p*-TSA, *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD), and acetonitrile for LC-MS/MS analysis with electrospray ionization and PbO<sub>2</sub> post-column oxidation. TMPD was used in place of HAH in this procedure to stabilize the dye compounds and prevent demethylation. Though post-column oxidation is not required for analysis by mass spectrometry, these authors noted improved sensitivity and reproducibility by converting leuco residues to the cationic dye compounds. Similar extraction procedures were applied to residue analysis methods by LC-MS/MS without post-column oxidation<sup>150</sup> and for HPLC-VIS/FL analysis.<sup>151</sup>

Several methods have been described for triphenylmethane compound analysis with a simpler extraction procedure using only McIlvaine buffer (pH 3) and acetonitrile extraction followed by cation exchange SPE clean-up for direct LC-MS/MS of dye and leuco compounds.<sup>152, 153</sup> These procedures did not include stabilizing compounds (i.e., HAH, TMPD, *p*-TSA) and eliminated the dichloromethane partitioning as well. Storey et al.<sup>154</sup> developed a procedure to extract fish with McIlvaine buffer (pH 4.5), EDTA, *p*-TSA, and TMPD for an LC-MS/MS residue screening method without additional liquid- or solid-phase clean-up. Van de Riet et al.<sup>155</sup> developed an extraction procedure to permit sensitive LC-MS/MS determination based on a simple tissue extraction using acetonitrile and perchloric acid, with dichloromethane and SPE clean-up.

Simple QuEChERS extractions have also been developed for triphenylmethane dye determinations as well. For example, regulatory methods were developed for MG and LMG residues in salmon and shrimp using acetic acid-modified acetonitrile for extraction and LC-TOF-MS for analysis. In the first case,<sup>156</sup> sodium chloride assisted the extraction from salmon and the extract was cleaned up with dispersive Bondesil-NH<sub>2</sub> sorbent. In the second,<sup>157</sup> anhydrous magnesium sulfate and sodium chloride were added to the shrimp extract, and the acetonitrile supernatant was cleaned up with dispersive PSA sorbent and additional magnesium sulfate. In another procedure,<sup>158</sup> fish was extracted with water, acetonitrile, and formic acid, while phase separation was assisted with anhydrous sodium sulfate and sodium acetate. A portion of the supernatant was collected and filtered for analysis by UHPLC-MS/MS. Extraction methods combined with LC-MS analysis have been recently reviewed in detail.<sup>159</sup>

Several of the early extraction methods<sup>123</sup> included overnight procedures, noting higher dye extraction yields from incurred tissues when overnight extraction was used. Hall et al.<sup>160</sup> studied the equilibrium for extraction of LMG and MG from incurred salmon muscle using acetonitrile and acetate buffer (pH 4.5). While LMG was quantitatively extracted by the first time point (1 hour), MG required approximately 16 hours reaching an equilibrium concentration in the extraction solvent. This group also studied the interconversion of MG and LMG during the extraction process. Very little LMG converted to MG, but up to 15% of MG converted to LMG. These results combined with metabolism studies have important consequences for regulatory analysis of triphenylmethane dyes in fish. Namely,



effective methods should include analytical procedures to detect the leuco compounds. Moreover, improved quantitative results will be achieved by preventing interconversion with compounds like HAH and TMPD. It was noted that incorporating matrix-matched calibration standards into the method along with isotopically labeled internal standards for each of the dye and leuco compounds will better model the complex extraction processes.

Hurtaud-Pessel et al.<sup>31</sup> developed a quantitative and confirmatory method in 2011 for MG, LMG, CV, LCV, and BG residue determination in trout by LC-MS/MS. The method was validated according to EU Decision No. (EC) 2002/657<sup>161</sup> with retention time matching and two selected reaction monitoring product ion transitions collected for each dye or leuco compound. In this simple procedure, fish tissue was extracted with HAH, acetonitrile, and magnesium sulfate without additional liquid- or solid-phase extraction step. Residue quantification required the use of four isotopically labeled internal standards for MG, LMG, CV, and LCV, and calibration was based on using extracted matrix-matched calibrants. As predicted by Hall,<sup>160</sup> the use of individual internal standards and matrix-matched calibrants provided excellent normalization of the complexity of dye residue analysis in fish. For regulatory analysis, the method performance for MG, LMG, CV, LCV, and BG was characterized by decision limit and detectability ( $CC\alpha$  and  $CC\beta$ ) at and below 0.5  $\mu\text{g/kg}$ , trueness ranging from 100% to 110% recovery, and precision of 10% RSD. Alternative instrument parameters were additionally described for the identification of the LBG analyte in incurred trout by UHPLC-LTQ-Orbitrap<sup>TM</sup>-MS.<sup>31</sup> The method was included in several proficiency testing studies conducted by the EU Reference Laboratory for EU Member States<sup>162</sup> and was the method suggested in a recent Food Emergency Response Network proficiency test conducted by the USFDA for state and federal laboratories in the USA. In 2012, the method was established as AOAC First Action Method 2012.25 for future consideration as an AOAC Official Methods of Analysis.<sup>163</sup> The method was independently studied and validated for salmon, catfish, shrimp, and tilapia with the method performance evaluated according to both USFDA and EU criteria for mass spectrometric confirmation of identity and method detection limit.<sup>28</sup> In 2015, the method was recommended by an Expert Panel Review for Final Action after review of the results of an AOAC Collaborative Study with participation from 14 regulatory, private, and academic laboratories from the USA, Canada, and France.<sup>32</sup> The AOAC Official Methods Board approved 2012.25 for Final Action Official Method status in February 2016.

### 9.5.2 Analytical Methods for Other Dyes in Seafood

Compared to the triphenylmethane dyes, there are few class-specific dye residue analysis methods for regulatory seafood monitoring. Some multi-class dye methods have been introduced in recent years, and these are described in the following section.

### 9.5.2.1 Phenothiazines

Like the triphenylmethane dyes, detection of MB by visible absorbance at 663 nm provides a sensitive and fairly selective analytical approach for dye residue determination. Nakagawa et al.<sup>36</sup> studied the uptake of MB by eels and found residues to be undetectable using a spectrophotometric analysis method. In this method, MB was extracted in *n*-butanol with zinc sulfate and analyzed spectrophotometrically. Kasuga et al.<sup>164</sup> developed a method to extract MB and MG residues from trout muscle with pH 3 McIlvaine buffer and acetonitrile with HPLC analysis.

In 1997, Turnipseed et al.<sup>37</sup> modified the earlier MG/LMG method by Roybal et al.<sup>133</sup> for the extraction of MB from catfish muscle. The procedure was based on initial tissue mixing with sodium acetate buffer (pH 4.5), *p*-TSA, and HAH to stabilize MB and limit demethylation to the azure metabolites. Acetonitrile was added as the extraction solvent and dispersive alumina added to adsorb fat. MB residues were partitioned into dichloromethane and then further isolated by solid-phase clean-up with alumina and weak cation exchange using a CBA SPE cartridge. The CBA SPE procedure permitted higher recoveries than the stronger propylsulfonic acid SPE used in the MG/LMG method.<sup>133</sup> MB residues were analyzed in fortified and incurred catfish extracts by HPLC with visible absorbance monitoring at 660–665 nm to yield 75–90% recovery over the concentration range 10–50 µg/kg. Though LMB could not be isolated for detection, it was converted to MB during the extraction and analysis. Azure B and other demethylated metabolites were present in the chromatography.<sup>37</sup>

The MB procedure developed by Turnipseed et al. formed the basis for MB extraction used in more recent methods for HPLC-VIS<sup>165</sup> and LC-MS/MS analysis.<sup>166</sup> For the LC-MS/MS analysis, selected reaction monitoring was used to monitor product ion transitions from both MB and LMB precursors (*m/z* 284 and 286, respectively) following electrospray ionization in positive ion mode. Though the researchers observed that LMB was not stable and easily oxidized to MB during the analysis, they were able to collect product ion spectra in full scan mode with weak signal for product 2 *m/z* units greater than the parent MB, which was indicative of the presence of LMB. For regulatory analysis, only the MB residue was validated over the concentration range 1–10 µg/kg for eel, toasted eel, and shrimp. Recovery ranged from 74% to 99% (%RSD < 17%) and the method detection limit was 0.1 µg/kg.

### 9.5.2.2 Xanthenes

Analytical methods for xanthene dyes in fish matrix are described in Section 9.5.3, “Multi-class Dye Residue Analysis Methods.” No class-specific methods for xanthene dye residue determination were found in the literature for fish muscle. One method described supercritical fluid extraction and solvent extraction from clay soils.<sup>167</sup> In this procedure, uranine, eosin Y lactone, phloxine B, Rose Bengal, and erythrosine B were separated on a C<sub>18</sub> HPLC column with ammonium acetate and acetonitrile gradient elution and spectrophotometric detection at 493, 525, and 546 nm.

### 9.5.2.3 Acridines

A residue determination method for acriflavine and proflavine was developed by Plakas et al.<sup>48</sup> in 1996 for catfish. Acidic methanol was used as the extraction solvent and residues were isolated with C<sub>18</sub> SPE cartridges. Quantitative analysis was performed by HPLC using a cyano column with absorbance measurement at 454 nm. The method was validated for fortified muscle over the concentration range 5–80 mg/kg. Recoveries were 86–95% with less than 6% RSD. This method was also used to extract metabolite compounds, though chromatographic separation was improved with a C<sub>8</sub> HPLC column.<sup>47</sup> Though not applied to fish muscle, a method was reported to determine acriflavine residue in waste water after isolation on Oasis<sup>®</sup> HLB SPE cartridges and analysis by LC-ESI-MS/MS in positive ion mode.<sup>168</sup>

Park et al.<sup>169</sup> recently developed an extraction and analysis procedure by LC-MS/MS for acriflavine and other veterinary drugs in pork, eggs, and milk. In this method, matrix was simply extracted with 0.1% formic acid and acetonitrile, the supernatant defatted with hexane and then evaporated, reconstituted, filtered, and analyzed by LC-MS/MS using a standard C<sub>18</sub> column and formic acid–acetonitrile elution gradient. This procedure<sup>169</sup> yielded significantly improved recovery compared to QuEChERS sample preparation. Intra-day recovery for acriflavine in pork matrix was 71% at the 5 µg/kg fortification concentration with an RSD of 15%. Kaufmann et al.<sup>170</sup> recently reported on the differences in identity confirmation using mass spectrometry with triple quadrupole or high-resolution techniques. Acriflavine was one of the many veterinary residues analyzed in beef liver matrix.

### 9.5.2.4 Azo Dyes

Methods were recently reported for the extraction and analysis of chrysoidine in fish matrix. Wang et al.<sup>51</sup> reported extraction of fish with methanol, solvent drying with anhydrous sodium sulfate, and clean-up with dispersive C<sub>18</sub> sorbent and magnesium sulfate. Extracts were derivatized and analyzed by GC-MS for confirmatory analysis and 81% recovery (4% RSD) of residues spiked at 10 µg/kg. Gui et al.<sup>171</sup> developed a method for chrysoidine in yellowfin tuna by LC-MS/MS. In this method, tuna was extracted with 1 M hydrochloric acid for an hour and neutralized to pH 7 with sodium hydroxide, and then residues were adsorbed onto Oasis HLB SPE cartridge for final elution, evaporation, and reconstitution. Tuna fortified with chrysoidine at 0.5 µg/kg yielded > 85% (<15% RSD). Reynolds et al.<sup>52</sup> extracted chrysoidine under basic conditions by adding sodium hydroxide to pangasius fish matrix and then extracting with ethyl acetate. A portion of the ethyl acetate was removed, evaporated, and dissolved in acetonitrile with formic acid and defatted with hexane prior to analysis by UHPLC-MS/MS. The method was validated according to Council Directive 2002/657/EC<sup>161</sup> with a 0.25 µg/kg limit of quantification.

In another azo dye analysis method, four Sudan dyes and their two metabolites were extracted from fish muscle, skin, and other animal products with acetonitrile, sodium sulfate, and ultrasound assistance. Extracts were defatted with hexane, residues collected onto basic alumina SPE cartridges, and the eluted dyes analyzed by LC-MS/MS.<sup>172</sup> Yamjala et al.<sup>173</sup> recently reviewed analytical methods for the determination of azo compounds used as food dyes.

### 9.5.3 Multi-class Dye Residue Analysis Methods

Many analytical methods to determine therapeutic dye residues in seafood products are class-specific methods, but as with the trend in veterinary residue analysis, larger multi-class methods began to emerge in 2008. Tarbin et al.<sup>14</sup> developed a quantitative multi-class LC-MS/MS residue method for triarylmethanes, phenothiazines, and a few compounds from the xanthene and phenoxazine classes (rhodamine 6G and Nile blue A) in seafood. This method included the most common and effective therapeutic dyes used in aquaculture (MG, CV, BG, and MB) and expanded the list to include other dyes that might be substituted for these to avoid regulatory detection, including pararosaniline, ethyl violet, the trinaphthylmethyl Victoria blue dyes, azure B, and new MB. Similar to other procedures,<sup>145</sup> the dyes were extracted from salmon using ammonium acetate buffer at pH 4.5, acetonitrile, and alumina followed by liquid–liquid extraction with dichloromethane, oxidation with DDQ, and cation exchange SPE. Because leuco metabolites are only available for MG and CV, the inclusion of a DDQ oxidation process drives leuco metabolites of triarylmethane and phenothiazine dyes to their chromic parent dye for simplified analysis.

Reyns et al.<sup>174</sup> recently expanded on this method for the detection of illegal therapeutic dye use in aquaculture. The 12 dyes included were the same as in the Tarbin et al. method,<sup>14</sup> though the extraction procedure was modified to extract eel matrix with acetonitrile and sodium acetate and eliminate the dichloromethane extraction. The DDQ oxidation was included to convert the leuco metabolites, and an additional CBA cartridge was coupled to the strong cation exchange solid-phase extraction procedure. This method was validated over the concentration range 0.25–1.0 µg/kg using UHPLC-MS/MS for analysis.

Xu et al.<sup>175</sup> reported a procedure for the extraction of MG, LMG, CV, LCV, MB, and three azure dye (A, B, and C) residues from silver carp with analysis by UHPLC-MS/MS. The extraction was based on the Roybal procedure,<sup>133</sup> though the choice of SPE sorbent was optimized. Strong cation exchange adsorbed MB and the azure dyes too strongly; weak cation exchange did not retain LMG and MG well. A combined C<sub>8</sub>-cation exchange cartridge (MCAX, Supelco) was found to be suitable for the clean-up of all the dye residues. Two product ion ratios were monitored for each dye to permit residue identification, and residue recovery was 75% or greater at the 0.5 µg/kg fortification concentration with RSD < 15%.<sup>175</sup>

Other multi-class methods have been developed with the intention of detecting dyes primarily used as food product dyes, some of which are also pharmacologically active dyes with possible aquaculture applications. Kirschbaum et al.<sup>176</sup> developed an HPLC-DAD method to test colored fish roe for permitted colorants from azo, xanthene, and triarylmethane dye classes. The dyes were extracted in aqueous ammonia, defatted with hexane, acidified to pH 2, and extracted onto polyamide powder for later elution and analysis. While this method was not intended to regulate therapeutic use of dyes in fish eggs, the method certainly is applicable for that purpose. Qi et al.<sup>177</sup> developed analyses for a similar group of permitted food dyes in fatty meat matrix with HPLC-DAD and LC-MS/MS. In this method, matrix was first extracted with hexane to remove fat and then extracted with ammoniated methanol with ultrasound assistance. Extracts were cleaned up with polymeric weak anion exchange cartridges. Sun et al.<sup>178</sup> reported a method for microwave-assisted extraction of 21 illegal dyes from meat and fish sausage. The 21 dyes included azo and xanthene dyes as well as triphenylmethanes and their leuco bases. Meat products were extracted in methanol/water with microwave irradiation for 5 minutes and then cooled and centrifuged. The dyes were absorbed onto C<sub>18</sub> SPE cartridges and then eluted for UHPLC-DAD absorbance analysis. All 21 compounds were separated using gradient elution with a pH 5 ammonium acetate buffer and acetonitrile and absorbance measurement at 254 and 600 nm. Limits of detection were 2 µg/kg or less and recovery ranged from 61% to 105% for the fish products.

#### **9.5.4 Bioanalytical Screening Methods**

In addition to chromatographic analyses coupled with spectrophotometric or mass spectrometric detection, the sensitivity and selectivity of immunoassay techniques make them useful for quickly screening large numbers of regulatory samples. Polyclonal antibodies have been reported for MG and LMG<sup>179</sup> and for LMG with cross-reactivity with MG and LCV.<sup>180</sup> ELISA test kits are also commercially available for screening fish products for MG/LMG (Bioo Scientific, EuroProxima), CV/LCV (Bioo Scientific), and MG or LMG (GlycoNex, Beacon Kits, Abraxis, Neogen).

Oplatowska et al.<sup>181</sup> produced a hybridoma cell line to generate a monoclonal antibody (mAb) with cross-reactivity for MG, CV, BG, methyl violet, methyl green, and Victoria blue R. This antibody did not bind the leuco metabolites, but LMG was effectively detected at 1 µg/kg in the rapid ELISA assay when DDQ oxidation was used in the extraction procedure for fish tissues. A similar procedure was used to produce a mAb for MG, CV, and oxidized leuco metabolites against a more effective carrier protein to enhance sensitivity and selectivity of the ELISA.<sup>182</sup> Jiang et al.<sup>183</sup> developed a hybridoma procedure to develop an antibody for LMG. The antibody had 100% cross-reactivity with MG, but did not bind CV or BG. Dong et al.<sup>184</sup> reported a non-competitive immunoassay based on phage anti-immune complex assay (PHAIA) detection for LMG. In

this technique, a specific peptide sequence was selected from a phage library with specific binding for a mAb–LMG complex. The assay was applied to tilapia extracts reduced with potassium borohydride to convert all MG residues to the leuco base. This PHAIA technique was reported to yield a 16-fold sensitivity enhancement for LMG detection compared to a competitive ELISA method with the same mAb. ELISA immunoassays have been developed to detect dyes from other classes including chrysoidine,<sup>185</sup> the Sudan azo dyes,<sup>186, 187</sup> and rhodamine B<sup>188</sup> residues in food products.

In other screening techniques, Stead et al.<sup>189</sup> developed an oligonucleotide RNA sequence as an aptamer to bind MG and provide a simple and sensitive fluorescence assay for the MG–aptamer complex. Xu et al.<sup>190</sup> developed a lateral flow immunoassay based on a colloidal gold-labeled mAb against MG. The assay had sufficient cross-reactivity with CV to permit rapid and sensitive detection of both residues on a test strip.

### 9.5.5 Other Notable Analytical Procedures

A number of analytical procedures have been designed to add extraction selectivity to the analysis of triphenylmethane dyes or concentrate the residues in the presence of the bulk fish extract. Several researchers<sup>191–193</sup> have developed molecularly imprinted polymer (MIP) materials for cartridge extraction to selectively adsorb dye compounds from fish extracts. One procedure provided sensitive detection for combined LMG/MG residues based on direct electrochemiluminescence analysis of the extract, where the highly selective MIP extraction was required to reduce matrix interference prior to analysis.<sup>192</sup> Dispersive sorbents for dye residues have been demonstrated using magnetic nanoparticles, where the dye-bound sorbent can be easily separated from the bulk fish extract by holding a magnet to the side of the extraction tube.<sup>194</sup> In recent research, MIPs were generated on the surface of magnetic nanoparticles for enhanced selectivity for MG extraction.<sup>195, 196</sup>

In other examples of the application of new solid sorbent materials for dye extraction, graphene oxide nanosheets were used for solid cartridge extraction of MG and LMG from fish extracts.<sup>197</sup> Magnetic graphene oxide nanocomposite material was used as a dispersive sorbent to concentrate MG residues extracted from trout for sensitive spectrophotometric analysis.<sup>198</sup> A graphene oxide sorbent was developed with an MIP coating for phloxine B residue extraction.<sup>199</sup>

Many novel sorbent materials based on graphene oxide have been studied for their ability to remove dyes from environmental effluents. Materials designed for effective adsorption of CV, MB, rhodamine B, acriflavine, and other dyes may have applications for fish extraction procedures as well.<sup>200–203</sup>

Liquid micro-extraction techniques have also been applied to concentrate dye residues from fish extracts prior to analysis. Dispersive liquid–liquid micro-extraction (DLLME) techniques were developed to concentrate triphenylmethane residues from fish and shrimp matrix into small volumes of immiscible

solvent<sup>204</sup> and ionic liquids.<sup>205</sup> In this research, DLLME permitted direct spectroscopic analysis of the dye residues from an optical cell without chromatographic separation. Direct analysis of MG, CV, and MB residues in fish extracts have been studied by surface-enhanced Raman scattering (SERS) as well.<sup>206–209</sup> Sorbent and liquid micro-extraction techniques were described in greater detail in Chapter 2.

## 9.6 Recent Trading Issues with Dye Alerts

In line with the countries' food laws, seafood inspection programs have been established across the world. These programs have been in place for more than 20 years in the largest seafood importing countries such as the Member States of the EU, the USA, Canada, and Japan. Regulatory agencies/administrations of importing countries (Table 9.1) are responsible for inspection of both the domestic farmed fish production and the imported aquaculture products. The veterinary drug residue content of this production and imports is carefully monitored in order to mitigate unintentional human exposures that may pose health risks. Seafood inspections also have to include checks for proper labeling and documentation, sensory evaluations, and laboratory screening for contaminants such as heavy metals, PCBs, toxins, and microbial pathogens.

The enforcement for the non-authorized dyes in aquaculture began in the early 2000s for the control of MG/LMG and was extended to CV/LCV soon after. They are still today the main officially controlled dye substances.

Love et al.<sup>210</sup> recently acquired sets of interesting data from the official inspection programs of several large seafood importing countries: EU members, the USA, Canada, and Japan. Through the extraction of data from several governmental websites, from published literature, and also from direct queries to governmental bodies, they examined the trends in the alerts for seafood contaminant violations over the period 2000–2009.

The records for EU seafood violations from domestic and imported products were available online from the RASFF portal.<sup>211</sup> USA seafood inspection data were acquired through a Freedom of Information Act (FOIA) request to the USFDA and included all tests for domestic and imported seafood from 1999 to 2006. Canada's Fish, Seafood and Production Division of the Canadian Food Inspection Agency (CFIA) provided non-compliant test results for seafood products containing veterinary drugs from 2000 to 2009. Japan's Ministry of Health, Labour and Welfare provided yearly totals for seafood inspections and violations online from 2004 to 2009 and positive tests for veterinary drugs from 2007 to 2009.<sup>212</sup>

Love et al.<sup>210</sup> examined the sets of non-compliant data collected from 2000 to 2009 in the major importing countries as a function of species of aquatic animals, exporting countries, drug types, and concentrations. The triphenylmethane dyes were one of the families of drugs included in their evaluation, considering primarily MG and CV, as these started to be controlled in the mid-2000s. Results

**Table 9.2** Percentage of veterinary drugs (dyes) violations collected over 2000 – 2009 period by seafood type and by inspection body.

Seafood types	Inspecting bodies			
	European Union 2001 – 2008	USA 2001 – 2006	Canada 2000 – 2009	Japan 2000 – 2009
Shrimp and prawns	None	None	3% malachite green	<1% malachite green
	100% other drugs	100% other drugs	97% other drugs	>99% other drugs
<i>n</i> *	545	27	239	205
Fin-fish	31% malachite green	77% malachite green	68% malachite green	None
	3% crystal violet	6% crystal violet	green	100% other drugs
	66% other drugs	17% other drugs	32% other drugs	
<i>n</i> *	211	81	435	97
Molluscan shellfish		None		8% malachite green
		100% other drugs		92% other drugs
				(75% chloramphenicol)
<i>n</i> *	0	1	0	13
Crabs	None	None	None	66% malachite green
	100% other drugs	100% other drugs	100% other drugs	green
	(56% chloramphenicol;	(chloramphenicol)	(98% nitrofurans)	33% other drugs
	42% nitrofurans)			(nitrofurans)
<i>n</i> *	36	40	45	3

*n*\*: number of violations recorded over the mentioned period.  
Source: David 2011 <sup>210</sup>. Reproduced with permission from American Chemical Society.



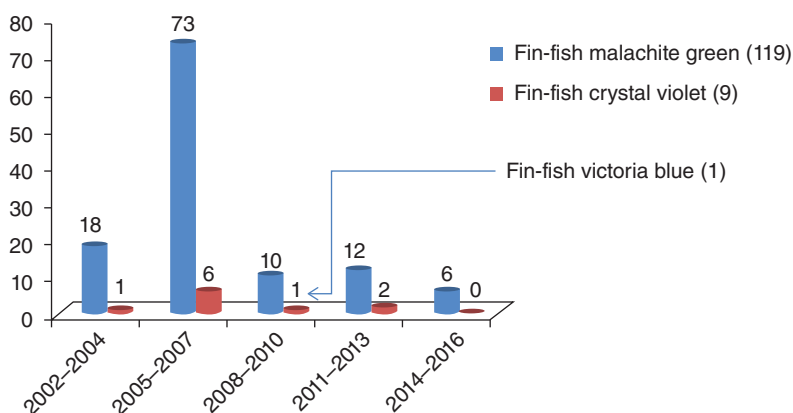
of their evaluation (Table 9.2) showed that fin-fish was the major species for violations with MG residues as reported by the EU ( $n = 65$ ), the USA ( $n = 62$ ), and Canada ( $n = 296$ ). However, a few cases of MG violative contamination were reported as well in shrimp and prawns in Canada ( $n = 7$ ) and Japan ( $n = 2$ ) and also in “molluscan shellfish” ( $n = 1$ ) and crabs ( $n = 2$ ) in Japan. A few cases of violations with CV residues were found in imported fin-fish in the EU ( $n = 6$ ) and the USA ( $n = 5$ ). According to Love et al.,<sup>210</sup> it was not systematically reported in the data extracted whether the violation was derived from a domestic sample or from an import sample.

A more recent survey was undertaken by the authors of this chapter through the EU RASFF portal.<sup>213</sup> The objective was to focus on the alerts exclusively derived from the dye residue violations in aquaculture products, that is, shrimp and prawns, fin-fish and “molluscan shellfish,” and cephalopods, respectively. Table 9.3 shows there were a total of 129 alerts that confirmed the presence of dye residues in these various aquaculture product consignments. This number was obtained from a long period spanning from 2002 to 2016. The alerts for dye residues accounted for more than 50% of the 247 fin-fish alerts in the EU (imports and domestic production altogether). According to the same table, very few of the 672 alerts derived from shrimp and prawn aquaculture were triggered due to the presence of MG residues (<1%). Finally, none of the four alerts in molluscan shellfish/cephalopod seafood imports/production were derived from

**Table 9.3** Percentage of veterinary drugs (dyes) violations by seafood type aquaculture. Extracted from the EU RASFF website over the period 2002–2016.<sup>213</sup>

Seafood types	Inspecting body: European Union RASFF period 2002–2016
Shrimp and prawns	0.3% malachite green 99.7% other drugs <sup>a)</sup>
$n^*$	672
Fin-fish	48.2% malachite green 3.6% crystal violet 0.4% Victoria blue 47.8% other drugs
$n^*$	247
Molluscan shellfish and cephalopod seafood	0.0% dyes (MG, CV, VB) 100% other drugs
$n^*$	4

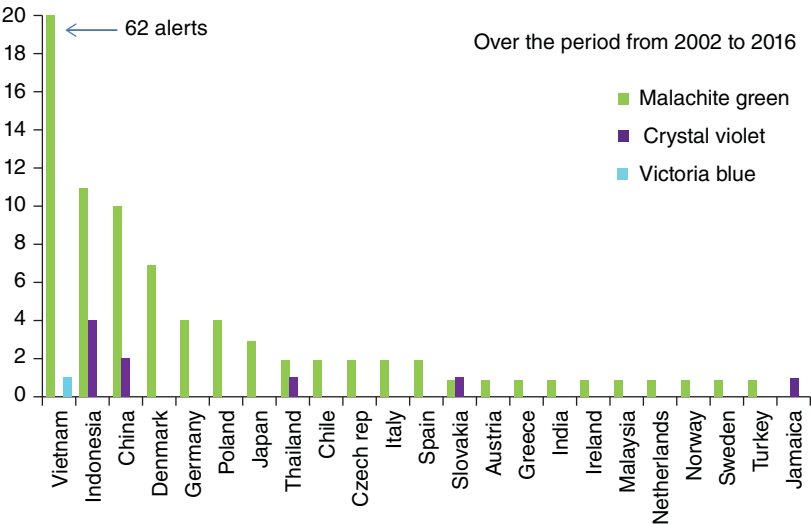
$n^*$ : number of violations recorded over the mentioned period.  
a) “Other drugs” include violations for chloramphenicol and nitrofurans.



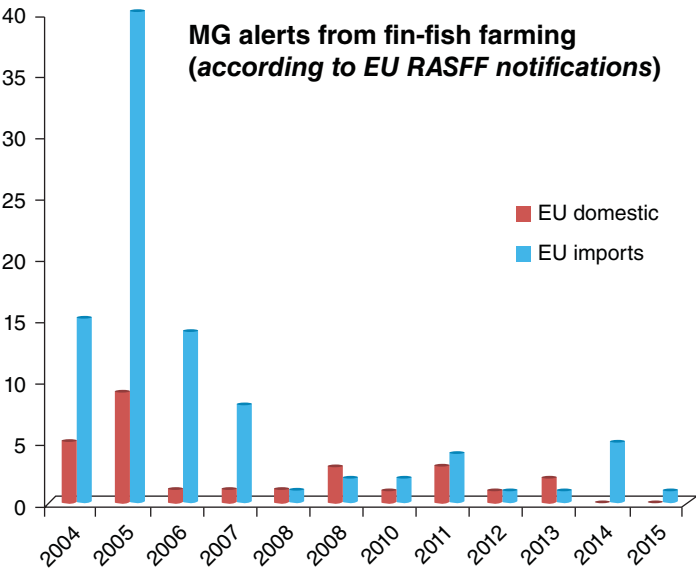
**Figure 9.4** EU alerts for dye residues in fin-fish aquaculture. Extracted from the EU RASFF website over the period 2002–2016.<sup>213</sup>

the presence of dye residues. Overall, the major dye substance found is MG. This is perhaps not unexpected because MG was the first dye to be used for antifungal and antimicrobial treatments in fish farms. It was also the first dye to be controlled in aquaculture production, whereas the official monitoring of CV actually started after the mid-2000s. It is worth highlighting one rather unexpected alert in Table 9.3 arising from the presence of Victoria blue residues in fin-fish fillets imported from Vietnam in 2010. In fact from the 129 alerts, 119 indicated MG contamination and 9 alerts showed CV contents. Over the 2002–2016 period, after a peak of alerts in the years 2005–2007 (73 MG alerts), the data displayed in Figure 9.4 clearly demonstrate that the dyes have not disappeared yet from the fin-fish farming industry and continue to potentially enter the food chain with two or three RASFF alerts per year in the more recent years as well.

Having now a closer look at the countries of origin of the fin-fish products subjected to the 129 alerts (Figure 9.5), the top three countries accounting for more than 10 alerts each are three Asian countries with quite large volumes of fish exports to the EU. Vietnam is the source for nearly 50% of the 129 alerts followed by Indonesia (15 alerts) and China (12 alerts). There are also a significant number of countries ( $n = 20$ ) that have been alerted (between 1 and 7 alerts each) due to the presence of MG or CV in their exported or domestic fin-fish products. Approximately half of these countries ( $n = 12$ ) are Member States of the EU which have been facing some safety issues with regard to their domestic fish farming production (i.e., Denmark, Germany, Poland). The other roughly half of the countries ( $n = 8$ ) are non-EU countries from Latin America and Asia (i.e., Japan, Thailand, Chile) which have been assigned a marketing authorization to export into the EU market in recognition of their implementation of an annual national residue monitoring plan demonstrating their ability to control their fish farming production in accordance with the EU regulations.<sup>99, 214</sup>



**Figure 9.5** EU alerts for dye residues in fin-fish aquaculture products sorted per countries of origin. Extracted from the EU RASFF website over the 2002–2016 period.<sup>213</sup>



**Figure 9.6** EU alerts for MG residues in fin-fish farming products sorted per year. Extracted from EU RASFF website over the period 2004–2015.<sup>213</sup>

It can also be clearly seen from Figure 9.6 that the larger number of alerts arises from the EU-imported products as compared to the EU domestic fin-fish production. It is clear that food safety and public health is still a big issue in aquaculture trading. Aquaculture products sold worldwide must be kept under sufficient control considering the various non-authorized chemical substances still available for fish/seafood farmers, including the dye substances and should start with serious control of MG itself.

## 9.7 Conclusions

The control of dye residues together with other regulatory prohibited/non-authorized or regulated chemicals in farmed fish and seafood products accounts for one of the public health concerns for this new century. The continuous rise of intensive and integrated aquaculture systems has to be seriously accompanied by appropriate controls and the various farming practices to be fully supported especially in developing countries. This area is acknowledged by the FAO to be one of the key elements to meet the urgent need worldwide to increase the efficiency and the volumes of food protein production in view of the ever faster growth of the human population.<sup>1</sup>

Facing this issue, most of the regulatory agencies in charge of food safety have developed programs to control these toxic chemicals in the food products derived from aquaculture. The ever-growing trading of food and in particular of fishery and farmed fish products has required governments to endorse adapted food laws in order to manage the risk of contaminated aquaculture and seafood products. Regulatory agencies of large exporting countries have been compelled to implement stricter conditions of use and even sometimes prohibition of these veterinary treatments in the intensive aquaculture practices developed in their countries over the past 20 years. As a result, there has been significant control deployed all around the world over the past 15 years. MG remains one of the key first issues to deal with for dye residue control in aquaculture around the world, together with a few other veterinary drugs of abuse such as nitrofurans and chloramphenicol, which are widely prohibited antibiotics.

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